Docket No. 23623-7078
GC Ref. No. 568-2-D1

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[X] **Pursuant to 37 C.F.R. § 1.10**, I hereby certify that this paper and all enclosures are being deposited with the **United States Postal Service as "Express Mail Post Office to Addressee" (EL710203395US)** on the date indicated below in an envelope addressed to the Assistant Commissioner for Patents, Box New Application, Washington D.C. 20231.

[ ] Pursuant to 37 C F R § 1.6(d), I hereby certify that this paper and all enclosures are being sent via facsimile on the date indicated below to the attention of Examiner _____ at Facsimile No. _____ at ____a.m./p.m.

Dated: February 12, 2002

Name of Person Certifying: Nancy Hine
Printed Name: Nancy Hine

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Davis et al.	Assignee:	Genencor International, Inc.
Filing Date:	Herewith	Examiner:	Not Yet Assigned
Serial No.:	Unknown	Group Art Unit:	Not Yet Assigned
Title:	**CHEMICALLY MODIFIED ENZYMES WITH MULTIPLE CHARGED VARIANTS**		

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please enter the following Preliminary Amendment for the above-identified patent application which is a divisional of Ser. No. 09/467,536, filed December 20, 1999, and claims the benefit of U.S. Provisional Patent Application No. 60/113,130, December 21, 1998.

IN THE SPECIFICATION

Please replace the paragraph beginning at 1:4 with the following rewritten paragraph:

—This application is a divisional of Ser. No. 09/467,536, filed December 20, 1999, pending, which claims the benefit of U.S. Provisional Patent Application No. 60/113,130, filed December 21, 1998, abandoned, the entire disclosures of which are hereby incorporated by reference in their entirety for all purposes.—

Please replace the paragraph beginning at 11:17 with the following rewritten paragraph:

—Figures 4A, 4B, 4C, and 4D illustrate altered specificity patterns relative to WT as the level of negative charge increases in N62C, L271C, S156C and S166C mutants and CMMs with suc-AAPF-*p*NA as the substrate: Figure 4A: The k_{cat}/K_Ms for N62C CMMs alternate at moderately reduced levels, 1.5- to 3.5-fold lower than WT, which are established by the initial mutation to N62C (R=H). Figure 4B: L217C CMMs show steady but lower levels of k_{cat}/K_M, 4- to 5.5-fold lower than WT, which are again established by the initial mutation to

cysteine. The exception is L217C-**c** which is only 2.5-fold lower than WT, possibly due to favorable binding of substrate to the phenyl ring of the aromatic side chain introduced by modification. Figure 4C: From the small reduction caused by mutation to S156C (R=H), k_{cat}/K_Ms decrease monotonically to 6-fold lower than WT for S156C-**d**. The k_{cat}/K_M of S156C-**e** is partially restored. Figure 4D: k_{cat}/K_M decreases only 2.5-fold upon mutation to S166C (R=H) but decreases dramatically to 11-fold lower than WT when the negatively charged sulfonatoethyl side chain **a** is introduced. In parallel to N62C and L217C CMMs, k_{cat}/K_M for S166C CMMs does not decrease further to any significant extent as the level of negative charge increases. R group key: a = sulfonatoethyl; b = 4-carboxybutyl; c = 3,5-dicarboxybenzyl; d = 3,3-dicarboxybutyl; e = 3,3,4-tricarboxybutyl.—

Please replace the paragraph beginning at 12:17 with the following rewritten paragraph:

—Figures 8A, 8B, 8C and 8D show altered specificity patterns for N62C (Fig. 8A), L271C (Fig. 8B), S156C (Fig. 8C), and S166C (Fig. 8D) CMMs: variations in ln (k_{cat}/K_M), with suc-AAPF-*p*NA as the substrate, for cysteine mutants and positively charged CMMs relative to WT. R group key: a = 2-aminoethyl; b = 2-(trimethylammonium)ethyl; c = 5,5-bis(aminomethyl)-3-oxo-hexyl; d = 2,2-bis(aminomethyl)-3-aminopropyl.—

Please replace the paragraph beginning at 14:25 with the following rewritten paragraph:

—Preferred serine hydrolases comprising this invention include the trypsin-chymotrypsin proteases, the subtilisin proteases, and the alpha/beta hydrolases. In a particularly preferred embodiment the enzyme is protease, more preferably a subtilisin (*e.g.* a *Bacillus lentus* subtilisin). Subtilisin is a serine endoprotease (MW ~27,500) which is secreted in large amounts from a wide variety of Bacillus species. The protein sequence of subtilisin has been determined from at least four different species of Bacillus (*see, e.g.,* Markland *et al.* (1971) pages 561-608 In: *The Enzymes*, ed. Boyer P. D., Acad Press, New York, Vol. III, pp.; Nedkov *et al.* (1983) *Hoppe-Seyler's Z. Physiol. Chem.* 364: 1537-1540). The three-dimensional crystallographic structure of subtilisin BPN' (from *B. amyloliquefaciens*) to 2.5 Å resolution has also been reported (Wright *et al.* (1969) Nature 221, 235-242; Drenth *et al.* (1972) *Eur. J. Biochem.* 26: 177-181. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, *et al.* (1972) *Biochemistry* 11: 2439-2449), product complexes (Robertus *et al.* (1972) *Biochemistry* 11: 4293-4303), and transition state analogs (Matthews *et al* (1975) *J. Biol. Chem.* 250: 7120-7126; Poulos *et al.* (1976) *J. Biol. Chem.* 251, 1097-1103), which have been reported have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisin (Philipp *et al.* (1983) *Mol. Cell. Biochem.* 51:5-32; Svendsen (1976) *Carlsbera Res. Comm.* 41: 237-291; Markland, *Id.*) as well as at least one report wherein the side chain of methionine at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer *et al.* (1965) *J. Biol. Chem.* 244: 5333-5338).—

Please replace the Table beginning at 33:21 with the following rewritten Table:

—**Table 1**. Kinetic Parameters[a] for Modified Enzymes

Entry	Enzyme	Pocket	R[c]	Level of Charge	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
1	SBL-WT	-	-	-	153 ± 4	0.73 ± 0.05	209 ± 15
2	N62C	S_2	H	0	174 ± 9	1.90 ± 0.20	92 ± 11
3			a	1	119 ± 4	0.93 ± 0.07	128 ± 11
4			b	1	106 ± 2	1.01 ± 0.05	105 ± 6
5			c	2	113 ± 7	1.00 ± 0.10	113 ± 13
6			d	2	90 ± 4	1.47 ± 0.14	61 ± 6
7			e	3	129 ± 3	1.46 ± 0.06	88 ± 4
8	L217C	S_1'	H	0	41 ± 1	0.80 ± 0.04	51 ± 3
9			a	1	89 ± 6	1.80 ± 0.20	50 ± 6
10			b	1	54 ± 1	1.03 ± 0.06	52 ± 3
11			c	2	69 ± 2	0.81 ± 0.06	85 ± 7
12			d	2	63 ± 2	1.65 ± 0.11	38 ± 3
13			e	3	55 ± 2	1.48 ± 0.08	37 ± 3
14	S156C	S_1	H	0	125 ± 4	0.85 ± 0.06	147 ± 11
15			a	1	87 ± 2	1.20 ± 0.07	73 ± 5
16			b	1	76 ± 1	1.08 ± 0.04	70 ± 3
17			c	2	61 ± 1	1.39 ± 0.10	44 ± 3
18			d	2	53 ± 1	1.67 ± 0.06	32 ± 1
19			e	3	74 ± 2	1.87 ± 0.08	39 ± 2
20	S166C		H	0	42 ± 1	0.50 ± 0.05	84 ± 9
21			a	1	25 ± 1	1.34 ± 0.08	19 ± 1
22			b	1	48 ± 1[b]	1.52 ± 0.06	31 ± 1[b]
23			c	2	47 ± 3	1.60 ± 0.20	29 ± 4
24			d	2	67 ± 2	2.26 ± 0.10	30 ± 2
25			e	3	76 ± 2	2.46 ± 0.11	31 ± 2

[a] Michaelis-Menten constants were measured at 25°C according to the initial rates method in 0.1 M Tris-HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, Suc-AAPF-*p*NA as the substrate.

[b] Based on total protein concentration. Certain inconsistencies between active enzyme concentration as determined by active site titration with PMSF (Hsia *et al.* (1996) *J. Anal. Biochem.* 242: 221-227) and total protein concentration have been reported for negatively charged mutants of SBL. These are characterized by sluggish fluoride ion concentration bursts and high rates of background PMSF hydrolysis. Active enzyme concentration values for S166C-b were low and gave rise to an anomalous value for kcat (270 ± 5 s-1). Consequently the value shown was calculated using total protein concentration as determined by absorbance at 280 nm (e280 = 23000 M-1 cm-1) (Grøn et al. (1990) Eur. J. Biochem. 194 :897-901). The KM value determined (1.52 ± 0.06 mM) is not concentration dependent.

[c] a = sulfonatoethyl; b = 4-carboxybutyl; c = 3,5-dicarboxybenzyl; d = 3,3-dicarboxybutyl; e = 3,3,4-tricarboxybutyl.—

Please replace the Table beginning at 48:25 with the following rewritten Table:

—**Table 2.** Kinetic Parameters[a] for Modified Enzymes

Entry	Enzyme	Pocket	R[b]	Level of Charge	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
1	SBL-WT	-	-	-	153 ± 4	0.73 ± 0.05	209 ± 15
2	N62C	S_2	H	0	174 ± 9	1.90 ± 0.20	92 ± 11
3			a	1	103 ± 5	1.00 ± 0.10	103 ± 11
4			b	1	73 ± 2	0.86 ± 0.05	85 ± 5
5			c	2	92 ± 3	1.06 ± 0.07	87 ± 6
6			d	3	98 ± 3	1.17 ± 0.08	84 ± 6
7	L217C	S_1'	H	0	41 ± 1	0.80 ± 0.04	51 ± 3
8			a	1	38 ± 1	0.64 ± 0.06	59 ± 6
9			b	1	43 ± 1	0.69 ± 0.03	62 ± 3
10			c	2	8.0 ± 0.2	2.94 ± 0.28	2.7 ± 0.3
11			d	3	23 ± 3	2.90 ± 0.16	7.8 ± 1.2
12	S156C	S_1	H	0	125 ± 4	0.85 ± 0.06	147 ± 11
13			a	1	90 ± 2	0.73 ± 0.04	123 ± 7
14			b	1	68 ± 2	0.74 ± 0.04	92 ± 5
15			c	2	64 ± 1	0.76 ± 0.04	85 ± 5
16			d	3	46 ± 1	0.81 ± 0.05	57 ± 4
17	S166C		H	0	42 ± 1	0.50 ± 0.05	84 ± 9
18			a	1	50 ± 1	0.68 ± 0.04	74 ± 5
19			b	1	33 ± 2	1.42 ± 0.13	23 ± 2
20			c	2	55 ± 2	1.27 ± 0.10	43 ± 4
21			d	3	9.3 ± 0.2	1.16 ± 0.05	8.0 ± 0.4

[a] Michaelis-Menten constants were measured at 25° C according to the initial rates method in 0.1 M Tris-HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, Suc-AAPF-*p*NA as the substrate.
[b] a = 2-aminoethyl; b = 2-(trimethylammonium)ethyl; c = 5,5-bis(aminomethyl)-3-oxo-hexyl; d = 2,2-bis(aminomethyl)-3-aminopropyl.—

Please replace the Table beginning at 63:22 with the following rewritten Table:

—**Table 3:** Kinetic Evaluation[(a)] of Altered S_1 Pocket Specificity

Entry	Enzyme	Substrate	K_M mM	k_{cat} s^{-1}	k_{cat}/K_M s^{-1} mM^{-1}
1	WT	suc-AAPF-pNA	0.73 ± 0.08	153 ± 4	209 ± 15
2	S166C-S-**a**	suc-AAPF-pNA	0.68 ± 0.04	50 ± 1	74 ± 5
3	S166C-S-**b**	suc-AAPF-pNA	1.34 ± 0.08	25.0 ± 0.7	19 ± 1
4	S166C-S-**c**	suc-AAPF-pNA	1.17 ± 0.06	23.1 ± 0.5	20 ± 1
5	S166C-S-**d**	suc-AAPF-pNA	1.6 ± 0.2	47 ± 3	29 ± 4
6	S166C-S-**e**	suc-AAPF-pNA	1.09 ± 0.07	82 ± 2	75 ± 5
7	S166C-S-**f**	suc-AAPF-pNA	0.70 ± 0.05	4.8 ± 0.1	6.90 ± 0.05
8	S166C-S-**g**	suc-AAPF-pNA	0.74 ± 0.07	29 ± 1	41 ± 4
9	S166C-S-**h**[(b)]	suc-AAPF-pNA	1.52 ± 0.06	48 ± 1	31 ± 1
10	S166C-S-**i**[(b)]	suc-AAPF-pNA	2.26 ± 0.10	67 ± 2	30 ± 2
11	S166C-S-**j**[(b)]	suc-AAPF-pNA	2.46 ± 0.11	76 ± 2	31 ± 2
12	WT	suc-AAPA-pNA	2.0 ± 0.1	17.7 ± 0.3	8.8 ± 0.4
13	S166C-S-**c**	suc-AAPA-pNA	0.8 ± 0.1	6.8 ± 0.3	9 ± 1
14	S166C-S-**e**	suc-AAPA-pNA	1.90 ± 0.03	6.8 ± 0.4	3.6 ± 0.6
15	S166C-S-**f**	suc-AAPA-pNA	1.90 ± 0.07	28.2 ± 0.4	14.8 ± 0.6
16	S166C-S-**g**	suc-AAPA-pNA	1.74 ± 0.04	9.65 ± 0.07	5.54 ± 0.3
17	WT	suc-AAPR-pNA	7.2 ± 0.7	0.16 ± 0.01	0.022 ± 0.002
18	S166C-S-**b**	suc-AAPR-pNA	3.4 ± 0.3	0.17 ± 0.01	0.050 ± 0.005
19	S166C-S-**d**	suc-AAPR-pNA	5.5 ± 1.1	0.68 ± 0.08	0.12 ± 0.03
20	S166C-S-**h**	suc-AAPR-pNA	8.2 ± 0.9	0.35 ± 0.02	0.041 ± 0.005
21	S166C-S-**i**	suc-AAPR-pNA	5.3 ± 0.5	0.43 ± 0.02	0.080 ± 0.008
22	S166C-S-**j**	suc-AAPR-pNA	5.2 ± 0.6	1.06 ± 0.07	0.20 ± 0.03
23	WT	suc-AAPE-pNA	4.4 ± 0.4	1.75 ± 0.08	0.40 ± 0.04
24	S166C-S-**a**	suc-AAPE-pNA	1.9 ± 0.1	14.5 ± 0.3	7.6 ± 0.4

(a) Michaelis-Menten constants were measured by the initial rates method in pH 8.6 Tris-HCl buffer at 25° C with suc-AAPF-pNA as the substrate.
(b) Based on total protein concentration. Certain inconsistencies between active enzyme concentration as determined by active site titration with PMSF (Hsia et al. (1996) J. Anal. Biochem. 242: 221-227) and total protein concentration have been reported for negatively charged mutants of SBL. These are characterized by sluggish fluoride ion concentration bursts and high rates of background PMSF hydrolysis. Active enzyme concentration values for S166C-S-h, S166C-S-i, and S166C-S-j were low and gave rise to anomalous values for kcat. Consequently the values shown were calculated using total protein concentration as determined by absorbance at 280 nm (ε_{280} = 23000 M-1 cm-1) (Grøn et al. (1990) Eur. J. Biochem. 194 :897-901). The K_M values determined are not concentration dependent.
(c) **a** = 2-aminoethyl; **b** = sulfonatoethyl; **c** = $CH_2C_6H_5$; **d** = $CH_2C_6H_4$-3,5-$(COO^-)2$; **e** = $CH_2(CH_2)_8CH_3$; **f** = $CH_2C_6H_{11}$; **g** = steroidyl; **h** = $CH_2(CH_2)_2CH_2COO^-$; **i** = $CH_2C(CH_3)(COO^-)_2$; j = $CH_2CH_2C(COO^-)_3$.—

AMENDMENTS TO SPECIFICATION

The specification has been amended to update the Cross-Reference to Related Applications Section.

The specification has been amended to correct spelling errors ("lentis" has been corrected to "lentus" and "amyloligoefaciens" has been corrected to "amyloliquefaciens").

Support for the amendments to Table 1 and the description of Figure 4 may be found at, for example, 38:15 – 43:28. Support for the amendments to Table 2 and the description of Figure 8 may be found at, for example, 51:8 – 57:29. Support for the amendments to Table 3 may be found at, for example, 61:25 - 62:13 and 64:15 – 66:26. The amendments therefore add no new matter.

IN THE CLAIMS

Prior to calculating the filing fee, please CANCEL claims 1-17, 35, 69, 71-84. Following entry of the Amendment, claims 18-34, 36-68, and 70 will be pending and at issue.

If the Examiner has any questions regarding this material, the Examiner is encouraged to Telephone Michael J. Shuster, Ph.D. at (415) 393-2651.

DATE: February 11, 2002

Respectfully submitted,

By: Michael J. Shuster

Michael J. Shuster, Ph.D.
Registration No.: 41,310

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Version Marked To Show Changes

IN THE SPECIFICATION

Please replace the paragraph beginning at 1:4 with the following rewritten paragraph:

This application [claims benefit under 35 U.S.C. §119 of provisional application USSN 60/113,130, filed on December 21, 1998, which is] is a divisional of Ser. No. 09/467,536, filed December 20, 1999, pending, which claims the benefit of U.S. Provisional Patent Application No. 60/113,130, filed December 21, 1998, abandoned, the entire disclosures of which are herein incorporated by reference in [its] their entirety for all purposes.

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Figures 4A, 4B, 4C, and 4D illustrate altered specificity patterns relative to WT as the level of negative charge increases in N62C, L271C, S156C and S166C mutants and CMMs with suc-AAPF-*p*NA as the substrate: Figure 4A: The k_{cat}/K_Ms for N62C CMMs alternate at moderately reduced levels, 1.5- to 3.5-fold lower than WT, which are established by the initial mutation to N62C (R=H). Figure 4B: L217C CMMs show steady but lower levels of k_{cat}/K_M, 4- to 5.5-fold lower than WT, which are again established by the initial mutation to cysteine. The exception is L217C-**c** which is only 2.5-fold lower than WT, possibly due to favorable binding of substrate to the phenyl ring of the aromatic side chain introduced by modification. Figure 4C: From the small reduction caused by mutation to S156C (R=H), k_{cat}/K_Ms decrease monotonically to 6-fold lower than WT for S156C-**d**. The k_{cat}/K_M of S156C-**e** is partially restored. Figure 4D: k_{cat}/K_M decreases only 2.5-fold upon mutation to S166C (R=H) but decreases dramatically to 11-fold lower than WT when the negatively charged sulfonatoethyl side chain **a** is introduced. In parallel to N62C and L217C CMMs, k_{cat}/K_M for S166C CMMs does not decrease further to any significant extent as the level of negative charge increases. R group key: a = sulfonatoethyl; b = 4-carboxybutyl; c = 3,5-dicarboxybenzyl; d = 3,3-dicarboxybutyl; e = 3,3,4-tricarboxybutyl.

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Figures 8A, 8B, 8C and 8D show altered specificity patterns for N62C (Fig. 8A), L271C (Fig. 8B), S156C (Fig. 8C), and S166C (Fig. 8D) CMMs: variations in ln (k_{cat}/K_M), with suc-AAPF-*p*NA as the substrate, for cysteine mutants and positively charged CMMs relative to WT. R group key: a = 2-aminoethyl; b = 2-(trimethylammonium)ethyl; c = 5,5-bis(aminomethyl)-3-oxo-hexyl; d = 2,2-bis(aminomethyl)-3-aminopropyl.

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Preferred serine hydrolases comprising this invention include the trypsin-chymotrypsin proteases, the subtilisin proteases, and the alpha/beta hydrolases. In a particularly preferred embodiment the enzyme is protease, more preferably a subtilisin (*e.g.* a *Bacillus* [***lentis***] ***lentus*** subtilisin). Subtilisin is a serine endoprotease (MW ~27,500) which is secreted in large amounts from a wide variety of Bacillus species. The protein sequence of subtilisin has been determined

from at least four different species of Bacillus (*see, e.g.,* Markland *et al.* (1971) pages 561-608 In: *The Enzymes,* ed. Boyer P. D., Acad Press, New York, Vol. III, pp.; Nedkov *et al.* (1983) *Hoppe-Seyler's Z. Physiol. Chem.* 364: 1537-1540). The three-dimensional crystallographic structure of subtilisin BPN' (from *B.* ***[amyloligoefaciens]*** ***amyloliquefaciens***) to 2.5 Å resolution has also been reported (Wright *et al.* (1969) Nature 221, 235-242; Drenth *et al.* (1972) *Eur. J. Biochem.* 26: 177-181. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, *et al.* (1972) *Biochemistry* 11: 2439-2449), product complexes (Robertus *et al.* (1972) *Biochemistry* 11: 4293-4303), and transition state analogs (Matthews *et al* (1975) *J. Biol. Chem.* 250: 7120-7126; Poulos *et al.* (1976) *J. Biol. Chem.* 251, 1097-1103), which have been reported have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisin (Philipp *et al.* (1983) *Mol. Cell. Biochem.* 51:5-32; Svendsen (1976) *Carlsbera Res. Comm.* 41: 237-291; Markland, *Id.*) as well as at least one report wherein the side chain of methionine at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer *et al.* (1965) *J. Biol. Chem.* 244: 5333-5338).

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Table 1. Kinetic Parameters[a] for Modified Enzymes

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13			e	3	55 ± 2	1.48 ± 0.08	37 ± 3
14	S156C	S_1	H	0	125 ± 4	0.85 ± 0.06	147 ± 11
15			a	1	87 ± 2	1.20 ± 0.07	73 ± 5
16			b	1	76 ± 1	1.08 ± 0.04	70 ± 3
17			c	2	61 ± 1	1.39 ± 0.10	44 ± 3

18		d	2	53 ± 1	1.67 ± 0.06	32 ± 1
19		e	3	74 ± 2	1.87 ± 0.08	39 ± 2
20	S166C	H	0	42 ± 1	0.50 ± 0.05	84 ± 9
21		a	1	25 ± 1	1.34 ± 0.08	19 ± 1
22		b	1	48 ± 1[b]	1.52 ± 0.06	31 ± 1[b]
23		c	2	47 ± 3	1.60 ± 0.20	29 ± 4
24		d	2	67 ± 2	2.26 ± 0.10	30 ± 2
25		e	3	76 ± 2	2.46 ± 0.11	31 ± 2

[a] Michaelis-Menten constants were measured at 25°C according to the initial rates method in 0.1 M Tris-HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, Suc-AAPF-*p*NA as the substrate.

[b] Based on total protein concentration. Certain inconsistencies between active enzyme concentration as determined by active site titration with PMSF (Hsia *et al.* (1996) *J. Anal. Biochem.* 242: 221-227) and total protein concentration have been reported for negatively charged mutants of SBL. These are characterized by sluggish fluoride ion concentration bursts and high rates of background PMSF hydrolysis. Active enzyme concentration values for S166C-b were low and gave rise to an anomalous value for kcat (270 ± 5 s-1). Consequently the value shown was calculated using total protein concentration as determined by absorbance at 280 nm (e280 = 23000 M-1 cm-1) (Grøn et al. (1990) Eur. J. Biochem. 194 :897-901). The KM value determined (1.52 ± 0.06 mM) is not concentration dependent.

[c] a = sulfonatoethyl; b = 4-carboxybutyl; c = 3,5-dicarboxybenzyl; d = 3,3-dicarboxybutyl; e = 3,3,4-tricarboxybutyl.

Please replace the Table beginning at 48:25 has been amended as follows:

Table 2. Kinetic Parameters[a] for Modified Enzymes

Entry	Enzyme	Pocket	R[b]	Level of Charge	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($s^{-1}mM^{-1}$)
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3			a	1	103 ± 5	1.00 ± 0.10	103 ± 11
4			b	1	73 ± 2	0.86 ± 0.05	85 ± 5
5			c	2	92 ± 3	1.06 ± 0.07	87 ± 6
6			d	3	98 ± 3	1.17 ± 0.08	84 ± 6
7	L217C	S_1'	H	0	41 ± 1	0.80 ± 0.04	51 ± 3
8			a	1	38 ± 1	0.64 ± 0.06	59 ± 6
9			b	1	43 ± 1	0.69 ± 0.03	62 ± 3
10			c	2	8.0 ± 0.2	2.94 ± 0.28	2.7 ± 0.3
11			d	3	23 ± 3	2.90 ± 0.16	7.8 ± 1.2
12	S156C	S_1	H	0	125 ± 4	0.85 ± 0.06	147 ± 11
13			a	1	90 ± 2	0.73 ± 0.04	123 ± 7
14			b	1	68 ± 2	0.74 ± 0.04	92 ± 5
15			c	2	64 ± 1	0.76 ± 0.04	85 ± 5

16		d	3	46 ± 1	0.81 ± 0.05	57 ± 4
17	S166C	H	0	42 ± 1	0.50 ± 0.05	84 ± 9
18		a	1	50 ± 1	0.68 ± 0.04	74 ± 5
19		b	1	33 ± 2	1.42 ± 0.13	23 ± 2
20		c	2	55 ± 2	1.27 ± 0.10	43 ± 4
21		d	3	9.3 ± 0.2	1.16 ± 0.05	8.0 ± 0.4

[a] Michaelis-Menten constants were measured at 25° C according to the initial rates method in 0.1 M Tris-HCl buffer at pH 8.6, 0.005% Tween 80, 1% DMSO, Suc-AAPF-*p*NA as the substrate.
[b] a = 2-aminoethyl; b = 2-(trimethylammonium)ethyl; c = 5,5-bis(aminomethyl)-3-oxo-hexyl; d = 2,2-bis(aminomethyl)-3-aminopropyl.

Please replace the Table beginning at 63:22 has been amended as follows:

Table [1]3: Kinetic Evaluation[(a)] of Altered S_1 Pocket Specificity

Entry	Enzyme	Substrate	K_M mM	k_{cat} s^{-1}	k_{cat}/K_M s^{-1} mM^{-1}
1	WT	suc-AAPF-pNA	0.73 ± 0.08	153 ± 4	209 ± 15
2	S166C-S-**a**	suc-AAPF-pNA	0.68 ± 0.04	50 ± 1	74 ± 5
3	S166C-S-**b**	suc-AAPF-pNA	1.34 ± 0.08	25.0 ± 0.7	19 ± 1
4	S166C-S-**c**	suc-AAPF-pNA	1.17 ± 0.06	23.1 ± 0.5	20 ± 1
5	S166C-S-**d**	suc-AAPF-pNA	1.6 ± 0.2	47 ± 3	29 ± 4
6	S166C-S-**e**	suc-AAPF-pNA	1.09 ± 0.07	82 ± 2	75 ± 5
7	S166C-S-**f**	suc-AAPF-pNA	0.70 ± 0.05	4.8 ± 0.1	6.90 ± 0.05
8	S166C-S-**g**	suc-AAPF-pNA	0.74 ± 0.07	29 ± 1	41 ± 4
9	S166C-S-**h**[(b)]	suc-AAPF-pNA	1.52 ± 0.06	48 ± 1	31 ± 1
10	S166C-S-**i**[(b)]	suc-AAPF-pNA	2.26 ± 0.10	67 ± 2	30 ± 2
11	S166C-S-**j**[(b)]	suc-AAPF-pNA	2.46 ± 0.11	76 ± 2	31 ± 2
12	WT	suc-AAPA-pNA	2.0 ± 0.1	17.7 ± 0.3	8.8 ± 0.4
13	S166C-S-**c**	suc-AAPA-pNA	0.8 ± 0.1	6.8 ± 0.3	9 ± 1
14	S166C-S-**e**	suc-AAPA-pNA	1.90 ± 0.03	6.8 ± 0.4	3.6 ± 0.6
15	S166C-S-**f**	suc-AAPA-pNA	1.90 ± 0.07	28.2 ± 0.4	14.8 ± 0.6
16	S166C-S-**g**	suc-AAPA-pNA	1.74 ± 0.04	9.65 ± 0.07	5.54 ± 0.3
17	WT	suc-AAPR-pNA	7.2 ± 0.7	0.16 ± 0.01	0.022 ± 0.002
18	S166C-S-**b**	suc-AAPR-pNA	3.4 ± 0.3	0.17 ± 0.01	0.050 ± 0.005
19	S166C-S-**d**	suc-AAPR-pNA	5.5 ± 1.1	0.68 ± 0.08	0.12 ± 0.03
20	S166C-S-**h**	suc-AAPR-pNA	8.2 ± 0.9	0.35 ± 0.02	0.041 ± 0.005
21	S166C-S-**i**	suc-AAPR-pNA	5.3 ± 0.5	0.43 ± 0.02	0.080 ± 0.008
22	S166C-S-**j**	suc-AAPR-pNA	5.2 ± 0.6	1.06 ± 0.07	0.20 ± 0.03
23	WT	suc-AAPE-pNA	4.4 ± 0.4	1.75 ± 0.08	0.40 ± 0.04
24	S166C-S-**a**	suc-AAPE-pNA	1.9 ± 0.1	14.5 ± 0.3	7.6 ± 0.4

(a) Michaelis-Menten constants were measured by the initial rates method in pH 8.6 Tris-HCl buffer at 25° C with suc-AAPF-pNA as the substrate.

(b) Based on total protein concentration. Certain inconsistencies between active enzyme concentration as determined by active site titration with PMSF (Hsia et al. (1996) J. Anal. Biochem. 242: 221-227) and total protein concentration have been reported for negatively charged mutants of SBL. These are characterized by sluggish fluoride ion concentration bursts and high rates of background PMSF hydrolysis. Active enzyme concentration values for S166C-S-h, S166C-S-i, and S166C-S-j were low and gave rise to anomalous values for kcat. Consequently the values shown were calculated using total protein concentration as determined by absorbance at 280 nm (ε_{280} = 23000 M-1 cm-1) (Grøn et al. (1990) Eur. J. Biochem. 194 :897-901). The K_M values determined are not concentration dependent.
(c) **a** = 2-aminoethyl; **b** = sulfonatoethyl; **c** = $CH_2C_6H_5$; **d** = $CH_2C_6H_4$-3,5-$(COO^-)2$; **e** = $CH_2(CH_2)_8CH_3$; **f** = $CH_2C_6H_{11}$; **g** = steroidyl; **h** = $CH_2(CH_2)_2CH_2COO^-$; **i** = $CH_2C(CH_3)(COO^-)_2$; j = $CH_2CH_2C(COO^-)_3$.